THE PRODUCTION OF AGGLUTININS IN RESPONSE TO ANTIGENS OF *ASCARIS LUMBRICOIDES* VAR. *SUUM*

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THE PRODUCTION OF AGGLUTININS IN RESPONSE TO ANTIGENS OF ASCARIS LUMBRICOIDES VAR. SUUM

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CHAPTER I

INTRODUCTION

Immunological reactions involving *Ascaris lumbricoides* var. *suum* have been demonstrated by *in vitro* serological tests. Precipitin antibodies[1] and complement-fixing antibodies[2] have been demonstrated against Ascaris antigens. It appears likely that other antibody types may be produced against Ascaris antigens, if appropriate techniques for their demonstration are devised. A literature search has failed to produce evidence of agglutinin production in experimental animals in response to antigens of Ascaris.

It is, therefore, the purpose of this investigation to determine the antigenicity of eggs and body fluid of *Ascaris lumbricoides* var. *suum* in the production of specific agglutinins, as measured by the agglutination of decoated Ascaris eggs. If egg agglutinins can be demonstrated experimentally, the application of such techniques may be of significance in the diagnosis of an infestation by this helminth.

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[1]Frances A. Coventry, "Hypersensitiveness to Helminth Proteins. II. Cutaneous and Precipitin Tests with Ascaris Extracts in Infected and Immunized Animals," *Journal of Preventive Medicine*, III (November, 1929), 43-62.

[2]W.K. Blackie, "Observations on the Immunity Response in Experimental Ascariasis in Rabbits," *Journal of Helminthology*, IX (June, 1931), 91-96.

CHAPTER II

HISTORY

Immunity to foreign proteins has been measured in a variety of ways involving *in vivo* and *in vitro* reactions. The demonstration of specific antibodies to antigenic compounds has been utilized as one type of measurement and, for the most part, involves serological tests. The resistance expressed by a living organism to a foreign antigen, as measured by survival or physiological responses, has also been utilized as an indication of an immune reaction.

Egg, larval, and adult tissues of various species of Ascaris have been studied to determine their relative antigenicity.[1] The response to Ascaris antigens has been demonstrated in experimental animals by the recovery of immune sera and the subsequent use of the antisera *in vitro* and *in vivo* tests.

Precipitin antibodies were reported by Schwartz,[2] using dried extracts of adult ascarids as antigenic material in an attempt to show relationships between different

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[1] J.F.A. Sprent and H.H. Chen, "Immunological Studies in Mice Infected with the Larvae of *Ascaris lumbricoides*. I. Criteria of Immunity and Immunizing Effect of Isolated Worm Tissues," *Journal of Infectious Diseases*, LXXXIV (March-April, 1949), 111-124.

[2] Benjamin Schwartz, "The Biological Relationships of Ascarids," *Journal of Parasitology*, XXX (September, 1920), 115-123.

species of Ascaris. The demonstration of this antibody was supported by the work of Coventry[1] and Canning.[2] More recently the production of precipitin antibodies has been reported by Campbell,[3] Gonzalez,[4,5] Sprent,[6] and Biagi.[7] Taliaferro[8] and Gonzalez[9] reported the production of a

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[1]Frances A. Coventry, "Hypersensitiveness to Helminth Proteins. II. Cutaneous and Precipitin Tests with Ascaris Extracts in Infected and Immunized Animals," Journal of Preventive Medicine, III (November, 1929), 43-62.

[2]Graeme A. Canning, "Precipitin Reactions with Various Tissues of Ascaris lumbricoides and Related Helminths," American Journal of Hygiene, IX (January, 1929), 207-226.

[3]Dan H. Campbell, "An Antigenic Polysaccharide Fraction of Ascaris lumbricoides (from hog)," Journal of Infectious Diseases, LIX (July-August, 1936), 266-280.

[4]Jose Oliver-Gonzalez, "Antigenic Analysis of the Isolated Tissues and Body Fluids of the Roundworm, Ascaris lumbricoides var. suum," Journal of Infectious Diseases, LXXII (May-June, 1943), 202-212.

[5]Jose Oliver-Gonzalez, "Functional Antigens in Helminths," Journal of Infectious Diseases, LXXIX (May-June, 1946), 232-237.

[6]Sprent, loc. cit.

[7]Franciso Biagi F., et al., "Value of a Skin Test and Precipitin Reaction in Diagnosis of Ascariasis," Journal of Infectious Diseases, CVII (September-October, 1960), 149-153.

[8]William H. Taliaferro, "The Mechanism of Acquired Immunity in Infections with Parasitic Worms," Physiological Reviews, XX (October, 1940), 469-489.

[9]Jose Oliver-Gonzalez, "Antigenic Analysis of the Isolated Tissues and Body Fluids of the Roundworm, Ascaris lumbricoides var. suum," Journal of Infectious Diseases, LXXII (May-June, 1943), 202-212.

precipitate at the excretory pores, mouth, and anal openings of the larval pig ascarid when placed in immune serum. The precipitate formed in this larval precipitin test is highly specific, and the titer is comparable to the 'ring' precipitin test.

Soulsby[1] reported that complement fixing antibodies were produced in response to Ascaris antigens but at a rather low titer. Later Soulsby[2] indicated that antibodies detectable by the complement fixation reaction follow the same general production pattern as heterophile antibodies. Heterophile antibodies produced in rabbits infected with Ascaris were found to be adsorbed by guinea pig kidney emulsions and fowl erythrocytes.

Serum-agar double diffusion experiments with Ascaris were carried out by Kagan.[3] He demonstrated that enteric Ascaris fluid has ten to fourteen antigens, unembryonated eggs have three to five antigens, and embryonated eggs have

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[1]E.J.L. Soulsby, "Studies on the Serological Response in Sheep to Naturally Acquired Gastro-intestinal Nematodes," Journal of Helminthology, XXX (July, 1956), 129-142.

[2]E.J.L. Soulsby, "Studies on the Heterophile Antibodies Associated with Helminth Infections. I. Heterophile Antibody in Ascaris lumbricoides Infection in Rabbits," The Journal of Comparative Pathology and Therapeutics, LXVIII (January, 1958), 71-81.

[3]Irving G. Kagan, "Serum-agar Double Diffusion Studies with Ascaris Antigens," Journal of Infectious Diseases, CI (July-August, 1957), 11-19.

two antigens. He also pointed out that the strongest antigens were non-lyophylized saline extracts of whole worms and enteric fluid.

The agglutination or clumping together of cells has been observed with high specificity among human erythrocytes, bacteria, parasitic protozoa, and in some cases non-living material. Isoagglutinins found in human sera are used in blood group determinations, and specific bacterial agglutinins are used in determining the antibody titer against specific bacterial species. Some of the parasitic protozoa that exhibit the phenomenon of agglutination are the Leishmanias and Trypanosomes.[1] Hemagglutination reactions using Ascaris extracts have been performed by Soulsby[2] and Gonzalez.[3] They have demonstrated that isoagglutinin-like antibodies can be produced in response to Ascaris antigens.

As far as can be determined, the agglutination reaction using decoated Ascaris eggs has not been studied. Based on the evidence that Ascaris antigens have been found

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[1]William H. Taliaferro, The Immunology of Parasitic Infections (New York, New York: The Century Company, 1929).

[2]E.J.L. Soulsby, "Studies on the Serological Response in Sheep to Naturally Acquired Gastro-Intestinal Nematodes," Journal of Helminthology, XXX (July, 1956), 129-142.

[3]Jose Oliver-Gonzalez, "Histopathological and Immunological Observations after Inoculation of Substances Isolated from the Muscle and Cuticle of Ascaris Lumbricoides," Journal of Infectious Diseases, CVII (July-August, 1960), 94-99.

to be highly specific as demonstrated by the precipitin test, and that antibodies which affect the Ascaris larvae seem to have been stimulated by antigens contained in the egg, it appears probable that agglutinins are produced in response to Ascaris eggs and body fluid antigens.

CHAPTER III

MATERIALS AND METHODS

Ascaris lumbricoides var. *suum* used in this investigation were obtained from the Iowa Packing Company, Des Moines, Iowa. The eggs of Ascaris were removed from the worms by placing the distal third of several uteri in a five hundred millliliter erlenmeyer flask containing fifty cubic centimeters of sterile saline and mincing the uteri with a 'Power-Stir' (Eberback Company) power mixer at medium speed for twenty minutes. The egg suspension was decanted into a fifty cubic centimeter centrifuge tube and centrifuged at two hundred and seventy-four times the acceleration of gravity for five minutes in a refrigerated centrifuge (International Equipment Company) at ten degrees centigrade. (All centrifugation mentioned hereafter was performed as stated above.) This mincing and centrifugation process was repeated until an estimated forty thousand eggs were recovered as calculated by volume. The eggs then were treated with five per cent sodium hypochlorite (Chlorox) for approximately twenty minutes whereby the outer protein coat was removed. The decoated eggs were washed and centrifuged three times using sterile saline, then placed in an all-glass hand homogenizer (Arthur H. Thomas Company), and allowed to settle for one hour. The saline was decanted

and the eggs crushed and diluted with sterile saline resulting in a crushed egg vaccine containing approximately four thousand crushed eggs per cubic centimeter. The egg vaccine was transferred to a sterile twenty millililter vaccine bottle, rubber stoppered, and placed in a hot water bath (Precision Scientific Company) of fifty-five degrees centigrade for thirty minutes. At the end of this period, the vaccine immediately was placed in the refrigerator at five degrees centigrade until needed. Twenty-four hours later one cubic centimeter of egg vaccine was withdrawn from the vaccine bottle by using a sterile two cubic centimeter syringe with a twenty-five gauge needle. One-half cubic centimeter of vaccine was mixed with ten cubic centimeters of sterile nutrient broth (Difco). Another one-half cubic centimeter was mixed with ten cubic centimeters of sterile, melted, cooled agar (Difco) for use in the preparation of a pour plate. The inoculated nutrient broth and the pour plate were placed in an incubator at thirty-seven degrees centigrade for forty-eight hours. The broth and pour plate were read at the end of a twenty-four period and a forty-eight hour period. Three colonies of non-spore forming bacilli were found on the pour plate indicating that the vaccine was not sterile. The vaccine was placed again in a hot water bath at fifty-five degrees centigrade for thirty minutes and cooled in the refrigerator. The culturing of the vaccine samples as above

indicated it to be sterile. The vaccine was stored at five degrees centigrade until needed.

Ascaris body fluid was obtained by using a sterile five cubic centimeter syringe with a twenty gauge needle. The needle was inserted in the coelomic cavity of the worms and the coelomic fluid withdrawn and centrifuged. The supernatant then was decanted into two sterile ten cubic centimeter vaccine bottles, rubber stoppered, and placed in a hot water bath at fifty-five degrees centigrade for one hour. After demonstrating the vaccine to be sterile by culturing techniques, the vaccine was stored at five degrees centigrade until needed.

Two young Himalayan rabbits each weighing approximately three kilograms were used as experimental animals. Rabbit number one was immunized with the body fluid vaccine, and rabbit number two was immunized with the crushed egg vaccine. Each rabbit was bled initially from the external marginal ear vein. Fifteen cubic centimeters of blood were collected from each animal in sterile fifty millliliter centifuge tubes, allowed to clot at five degrees centigrade for several hours, 'ringed', and allowed to stand overnight. The blood was then centrifuged at one hundred and ninety-two times the acceleration of gravity for one-half hour at ten degrees centigrade. The sera were decanted into two sterile ten cubic centimeter vaccine bottles, rubber

stoppered, and stored at five degrees centigrade. These sera were the controls.

Each rabbit was inoculated intravenously or in one instance intraperitoneally according to the injection schedule shown in Table I. Sterile two cubic centimeter syringes with twenty-seven gauge needles were used. Aseptic techniques were strictly enforced. After the last injection, each rabbit was bled from the external marginal ear vein to obtain ten cubic centimeters of blood. The blood was placed in two sterile twenty cubic centimeter tubes, 'ringed', and put in the refrigerator overnight. The next morning by using the sera obtained from the test bleeding, the 'ring' precipitin test was executed to detect the presence of precipitin antibodies. Micro tubes approximately thirty-five millimeters in length and two and one-half millimeters in width were used.

Duplicate precipitin tests were performed using ten-fold dilutions of egg and body fluid antigens against control sera and homologous antisera. Precipitin antibodies were demonstrated in titers of 1:1000 with the body fluid vaccine and 1:10 with the egg vaccine. The precipitin test results are summarized in Tables II and III.

Upon the demonstration of precipitin antibodies, each rabbit was sacrificed by the injection of Nembutal into the external marginal vein. A heart puncture, using a sterile fifty milliliter syringe with a twelve gauge needle, was used

TABLE I

INJECTION SCHEDULE FOR ASCARIS EGG AND BODY FLUID ANTIGENS IN EXPERIMENTAL RABBITS

Day	Amount Injected			
	Rabbit with Egg Vaccine		Rabbit with Body Fluid Vaccine	
1	Intra-	1 cc	Intra-	1 cc
2	venous	-	venous	-
3		1 cc		½ cc
4		-		-
5		1 cc		1 cc
6		-		-
7		-		-
8		1 cc		½ cc
9		-		-
10		-		-
11		-		-
12		1 cc		1 cc
13		-		-
14		-		-
15		-		-
16		-		-
17		1 cc		½ cc
18		-		-
19		-		-
20		-		-
21		-		-
22	Intraper-	1 cc		½ cc
23	itoneal	-		-
24		-		-
25		-		-
26		Bleed		Bleed
Totals		7 cc		5 cc

TABLE II

PRODUCTION OF PRECIPITIN ANTIBODIES IN RABBITS IN RESPONSE TO EGG AND BODY FLUID ANTIGENS OF *ASCARIS LUMBRICOIDES* VAR. *SUUM*

Antigen dilution	Immune sera from rabbit #1 injected with body fluid antigen		Immune sera from rabbit #2 injected with egg antigen	
	Test 1	Test 2	Test 1	Test 2
Undilute	+	+	+	+
1:10	+	+	+	+
1:100	-	+	-	-
1:1000	+	-	-	-

+ = precipitate
- = no precipitate

TABLE III

PRECIPITIN TEST RESULTS ON NORMAL SERA OBTAINED FROM RABBITS BEFORE INJECTION OF EGG AND BODY FLUID ANTIGENS OF *ASCARIS LUMBRICOIDES* VAR. *SUUM*

Antigen	Normal sera from rabbit #1 before injection of body fluid antigen	Normal sera from rabbit #2 before injection of egg antigen
Undilute	-	-
Saline (0.85% NaCl)	-	-

+ = precipitate
- = no precipitate

to withdraw fifty millliliters of blood from each rabbit. The blood was placed in two sterile one hundred and twenty-five millliliter erlenmeyer flasks, 'ringed', and stored in the refrigerator at five degrees centigrade. The sera obtained were decanted into two sterile fifty millliliter centrifuge tubes and centrifuged at eight hundred and fifteen times the acceleration of gravity for fifteen minutes. The supernatant was decanted into two sterile one hundred and fifty centimeter test tubes with cotton stoppers and stored in the refrigerator at five degrees centigrade until needed.

Fresh Ascaris eggs were obtained on the day the agglutination tests were to be carried out. The eggs were recovered and decoated in the same manner as described previously, obtaining a concentration of approximately four thousand decoated eggs per cubic centimeter suspended in sterile saline. All the equipment to be used for the agglutination test were prepared as follows: microscope slides were acid cleaned, rinsed in distilled water, and dried; one millliliter pipettes were sterilized in the autoclave; hanging drop slides and cover slips were washed in detergent, rinsed in distilled water, and dried.

The agglutination test was performed using standard microscope slides and hanging drop slides. The hanging drop method was adopted as the method of choice since results were easier to read. One-tenth cubic centimeter

of suspended decoated Ascaris eggs was placed on a clean cover slip and mixed with one-tenth cubic centimeter of undilute immune serum using sterile toothpicks. Petroleum jelly (Vaseline) was applied in a thin layer around the depression chamber of a hanging drop slide. The depression chanber was placed over the cover slip containing the suspension of eggs and serum, and the slide was quickly inverted and examined microscopically for agglutination of the eggs. Two-fold serial dilutions were made with the immune sera to determine agglutinin titer and compared with normal sera and saline controls.

CHAPTER IV

RESULTS AND INTERPRETATION OF DATA

The experimental production of agglutinin antibodies in response to body fluid and egg antigens of Ascaris lumbricoides var. suum, can be demonstrated under the conditions present in this investigation.

The normal distribution of Ascaris decoated eggs in a saline hanging drop preparation was studied at a room temperature of thirty-two degrees centigrade, and humidity of about seventy per cent. Variation in temperature and humidity were not observed to influence the distribution of decoated Ascaris eggs in this type of preparation. One-tenth cubic centimeter of suspended eggs from a saline solution containing approximately four thousand eggs per cubic centimeter were observed. It was found that if the eggs were well-mixed, the distribution was uniform upon immediate observation. Very rarely were eggs observed adhering to each other upon immediate examination. A saline egg suspension was observed for thirty minutes and there was no indication of clumping for the first fifteen minutes. During the second fifteen minute period, the eggs started to collect in the bottom of the drop but they did not clump. Because of the ease in reading the reaction, all agglutination tests were read between eight and ten minutes after the addition of immune sera. No agglutination

was detected in the normal serum control from rabbit number one and from rabbit number two. The control tests for the agglutination reaction of decoated eggs of Ascaris lumbricoides var. suum are summarized in Table IV.

Two-fold serial dilutions of immune sera were prepared from rabbit number one (which had been immunized with the body fluid vaccine) and from rabbit number two (which had been immunized with the egg vaccine). Four separate tests were conducted with each serial dilution. A consistent antibody titer of one to sixteen in both immune sera were observed in all four tests. The highest agglutinin titer shown in serum produced in response to the body fluid antigen was one to sixty-four, whereas the highest agglutinin titer shown in serum produced in response to the egg vaccine was one to thirty-two. The results of the agglutination reaction using decoated eggs of Ascaris lumbricoides var. suum with immune sera are summarized in Table V.

Figure I shows photographs of Ascaris eggs in the presence of normal sera produced under the conditions present in this investigation. Photograph A shows decoated eggs in sterile saline solution, at a magnification of one hundred times. Photograph B was taken of decoated eggs plus normal serum from rabbit number one, at a magnification of thirty-five times. Photograph C shows the agglutination

TABLE IV

CONTROL TESTS ON TWO-FOLD SERIAL DILUTIONS OF NORMAL SERA FROM EXPERIMENTAL RABBITS USING DECOATED EGGS OF ASCARIS LUMBRICOIDES VAR. SUUM

Experimental Animal	Normal Rabbit Sera Dilution						
	Undilute	1:2	1:4	1:8	1:16	1:32	1:64
Rabbit #1	-	-	-	-	-	-	-
Rabbit #2	-	-	-	-	-	-	-

- = No agglutination

TABLE V

RESULTS OF THE AGGLUTINATION REACTION INVOLVING DECOATED EGGS OF *ASCARIS LUMBRICOIDES* VAR. *SUUM* AND IMMUNE SERA FROM RABBITS INJECTED WITH *ASCARIS* BODY FLUID AND CRUSHED EGG ANTIGENS

	Rabbit #1 Injected with Body Fluid				Rabbit #2 Injected with Crushed Eggs			
Sera Dilutions	Test 1	Test 2	Test 3	Test 4	Test 1	Test 2	Test 3	Test 4
Undilute	+	+	+	+	+	+	+	+
1:2	+	+	+	+	+	+	+	+
1:4	+	+	+	+	+	+	+	+
1:8	+	+	+	+	+	+	+	+
1:16	+	+	+	+	+	+	+	+
1:32	-	+	+	+	+	+	-	-
1:64	-	-	+	-	-	-	-	-

+ = Agglutination

- = No agglutination

Figure 1. Agglutination Reactions of Decoated eggs of Ascaris lumbricoides var. suum.

A. Eggs suspended in sterile saline (100x).

B. Eggs suspended in normal control serum (35x).

C. Eggs mixed with immune serum from rabbit immunized with egg vaccine (35x).

D. Eggs mixed with immune serum from rabbit immunized with body fluid vaccine (35x).

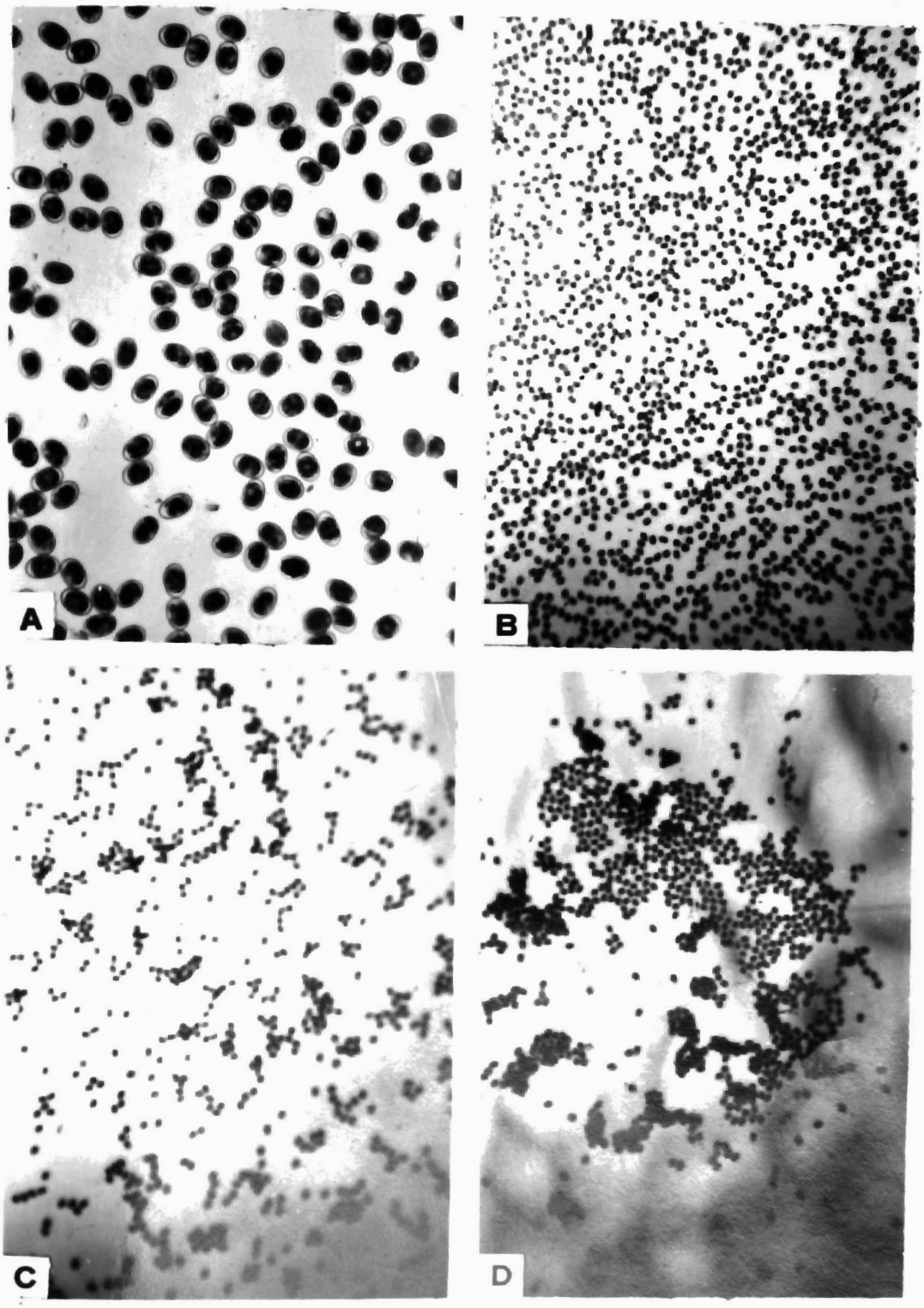

FIGURE 1

of Ascaris decoated eggs when mixed with immune serum produced in response to Ascaris egg vaccine. Photograph D is charactistic of the agglutination reaction of Ascaris decoated eggs when mixed with immune serum produced in response to Ascaris body fluid vaccine.

The process of agglutination of the suspended decoated eggs in immune serum was observed carefully by microscopic examination. In the presence of immune sera, the eggs slowly appeared to come closer to each other. Chains of eggs began to appear which were followed by clumping in small groups and then clumping in larger groups with the number of eggs per group seldom exceeding thirty in number. As the agglutination reaction appeared, the slide was agitated without disrupting the hanging drop demonstrating that the egg clumps were firm ones.

The agglutination reaction as demonstrated with decoated Ascaris eggs demonstrates that agglutinins can be produced in response to egg and body fluid of Ascaris antigens. Ascaris enteric fluid was reported by Kagan[1] to have ten to fourteen antigens, and unembryonated eggs were reported to have three to five antigens. The present study indicates that an agglutinogen is one additional type of antigen contained in body fluid and unembryonated eggs of

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[1]Kagan, loc. cit.

Ascaris. The work of Soulsby[1] and Gonzalez[2] in demonstrating that isoagglutinin-like antibodies can be produced in response to Ascaris antigens is partially supported by this study.

The demonstration of agglutinins produced in response to Ascaris antigens as reported in this investigation should be studied under conditions which differ from this study. It is suggested that the crossing of several Ascaris antigens with heterologous antisera should be investigated. An additional study which might be carried out is to determine the time required for agglutination as measured by the number of egg clumps formed after the addition of immune sera.

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[1]Soulsby, loc. cit.

[2]Gonzalez, loc. cit.

CHAPTER V

SUMMARY

Studies of immunological reactions with Ascaris lumbricoides var. suum have been carried out by a number of investigators involving in vitro and in vivo tests. As far as can be determined agglutinin antibodies have not been demonstrated. It seemed likely that agglutinins may be produced in experimental animals if appropriate techniques for their demonstration were devised.

Two rabbits were immunized with body fluid and egg antigens of Ascaris lumbricoides var. suum at various intervals for a three and one-half week period. The rabbits then were sacrificed and the immune sera recovered.

Decoated Ascaris eggs were suspended in sterile saline. One-tenth cubic centimeter of immune serum was added to one-tenth cubic centimeter of suspended egg solution. The demonstration of agglutinin antibodies against the body fluid and egg antigens of Ascaris was accomplished by the addition of immune sera to a saline egg suspension in a hanging drop preparation. The highest agglutinin titer demonstrated against the Ascaris egg antigen was one to thirty-two. The highest agglutinin titer demonstrated against the Ascaris body fluid antigen was one to sixty-four.

The results obtained from this study indicate that

agglutinins can be produced in response to body fluid and egg antigens of Ascaris lumbricoides var. suum.

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